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Cost-effective technique for preserving entomopathogenic fungi

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Abstract

The storage of entomopathogenic fungi (EPF) with minimal loss of viability and pathogenicity is crucial for their conservation and the maintenance of fungal biodiversity in collections. The aim of this study was to describe a simple and cost-effective method for the preservation of EPF using filter paper. The viability of nine EPF species was tested after storage at 4 °C using a modified filter paper method. *Beauveria* sp. and *Metarhizium* spp. showed the best results, with some strains surviving up to nine and seven years, respectively. For most of the other species evaluated, except *Cordyceps* sp., the method was effective. Additionally, the virulence of *Beauveria* sp., *M. anisopliae,* and *M. hybridum* against *Tenebrio molitor* larvae was assessed after storage and compared to *in vitro* viability, showing virulence levels ranging from 63% to 100% in preserved isolates. Viability can thus serve as an indirect parameter of virulence, as germ tube formation facilitates cuticle penetration, making it a critical factor when selecting an appropriate preservation method. This method facilitates the preservation of EPF, contributing to the maintenance of strains in collections and the conservation of fungal diversity for use in biological control.

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Introduction

The preservation of a fungal culture collection is a task that demands constant dedication and surveillance. It requires knowledge of the morphological, physiological, and biochemical characteristics of fungi, as well as their requirements for preservation methods[1]. To date, several techniques have been used to preserve fungal cultures. Cryopreservation, which involves storing spores or mycelia at ultra-low temperatures (typically in liquid nitrogen or −80 °C freezers), ensures long-term viability and genetic stability. However, it requires specialized equipment, is costly, and is vulnerable to power outages that may compromise sample integrity[2]. Lyophilization, or freeze-drying, removes water from the fungal material under vacuum after freezing, facilitating storage, and transport. However, it is an expensive technique, which can limit its routine use. The process is generally effective for most conidial fungi, but each isolate should be evaluated individually[2]. These limitations highlight the need for alternative, low-cost methods, such as silica gel, sterile water, and mineral oil preservation, that are accessible to laboratories with limited resources[3]. In addition, some other preservation methods are not compatible with entomopathogenic fungi (EPF) since, for example, repeated subculturing of EPF on artificial media results in loss of their pathogenicity, virulence, and/or sporulation[2]. No preservation method can be universally applied to all fungi, and intraspecific variability makes it impossible to apply standard protocols, even at the species level[4]. Humber[2] considered that the choice of different preservation techniques is often difficult, but should be based on the suitability of a given method to the needs of a laboratory to preserve fungi and on the technological and financial capabilities available. Ayala-Zermeño et al.[5] reported that it is advisable to choose more than one preservation method for the biological material of interest and thus reduce the possibility of all cultures losing viability and other characteristics.

The collection of fungal pathogens and symbionts of insects and other arthropods of the Center for Parasitological and Vector Studies (CEPAVE), National University of La Plata (UNLP) – National Scientific and Technical Research Council (CONICET), La Plata, Argentina, is unique because it preserves *in vivo* and *in vitro* cultures of fungal pathogens[6]. This culture collection is open for research, teaching, consulting services, and strain deposit[7]. The management and operation of the collection must follow the quality guidelines established by the Latin American Federation for Culture Collections (FELACC) and the World Federation of Culture Collections (WFCC); therefore, it is necessary to develop adequate protocols for the preservation of each species of EPF. The present study describes a simple and economical technique for the preservation of EPF cultures using filter paper. This method was adapted and modified on the basis of a previous publication[8], and has been used in culture collection for ten years with positive results, maintaining the purity, viability, and pathogenicity of cultures, as well as the morphological characteristics of cells[7].

Materials and methods

Initial cultures

The fungal isolates were obtained from pure cultures originating from insect hosts or soil (Table 1). Cultures were isolated on potato dextrose agar (PDA) or Sabouraud dextrose agar supplemented with 1% yeast extract (SDAY 1%), depending on the species, and incubated at 25 ± 1 °C in darkness for ten to 15 d. They were used once optimal purity and sporulation were achieved.

Storage setup

To perform this process, Whatman filter paper no. 4 was cut into 15 mm × 5 mm strips and sterilized twice in an autoclave at 120 °C for 20 min on two consecutive cycles, followed by drying in an oven at 70 °C for 24 h. In this way, the filter paper was free of potential

Table 1. Evaluation of the quality of cultures, viability, pathogenicity, and purity after recovery from preservation on the filter paper method.

Specie/strain	Insect host/substrate	Locality	Year of preservation[a]	Time (years)[b]	Mycelial growth/ sporulation[c]	Viability	Pathogenicity (% sporulation)[d]
Akanthomyces lecanii CEP054	Hemiptea: Aleyrodidae	Buenos Aires	2018	3	C	–	–
Akanthomyces muscarius CEP063	Hemiptera: Delphacidae	Buenos Aires	2014	7	+++	95	NT
Akanthomyces muscarius CEP182	Hemiptera: Aphididae	Buenos Aires	2015	6	+++	100	NT
Beauveria bassiana CEP317	Soil	Buenos Aires	2012	9	+++	70	95 (83)
Beauveria bassiana CEP570	Hymenoptera: Formicidae	Buenos Aires	2016	5	+++	77	98 (95)
Beauveria bassiana CEP588	Coleoptera	Buenos Aires	2016	5	+++	94	63 (77)
Beauveria sp. CEP606	Hymenoptera: Formicidae	Misiones	2017	4	+++	99	100 (94)
Cordyceps fumosorosea CEP307	Hemiptea: Aleyrodidae	Buenos Aires	2018	3	+	–	–
Cordyceps fumosorosea CEP371	Hemiptera: Aphididae	Buenos Aires	2018	3	+++	21	NT
Cordyceps javanica CEP107	Hemiptera: Aleyrodidae	Buenos Aires	2018	3	–	–	–
Cordyceps sp. CEP708	Lepidoptera	Jujuy	2018	3	–	–	–
Lecanicillium aphanocladii CEP556	Araneae: Nemesiidae	Buenos Aires	2018	3	+++	100	NT
Metarhizium anisopliae CEP399	Soil	San Juan	2019	2	++	79	100 (85)
Metarhizium anisopliae CEP404	Soil	San Juan	2014	7	+++	91	80 (75)
Metarhizium hybridum CEP085[e]	Hemiptera: Cercopidae	Buenos Aires	2019	2	+++	97	100 (100)
Metarhizium hybridum CEP160[e]	Hemiptera: Cercopidae	Corrientes	2020	2	+++	95	85 (65)
Purpureocillium sp. CEP643	Hemiptera: Cicadidae	Jujuy	2017	4	+++	82	NT
Purpureocillium sp. CEP780	Hemiptera: Pentatomidae	Misiones	2018	3	+++	35	NT

[a] Year conidia were preserved in filter paper. [b] Time of preservation of conidia in filter paper (years). [c] Mycelial growth and sporulation. Normal mycelial growth and sporulation (+++). Scarce mycelial growth and sporulation (++). Normal mycelial growth, and there is no sporulation (+). No growth (–). Contamination by bacteria or fungi (C). Not tested with that species (NT). [d] Pathogenicity is expressed as virulence (% larval mortality); sporulation (%) of cadavers is shown in parentheses. [e] *Metarhizium hybridum* is a recently described species[11]. In this study, several isolates were identified as *M. hybridum* based on molecular data, and their corresponding GenBank accession numbers reported in the study by Lozano et al.[12].

contaminants. All subsequent procedures were carried out under a laminar flow hood. Using sterile forceps, the sterilized filter paper strips were placed on sporulated fungal cultures for 60 min to allow conidia adhesion (Fig. 1). The filter paper strips were then removed and transferred to open Petri dishes (100 mm diameter), where they were dried for 2 h under laminar air-flow. Following this, two to four strips were placed into 1.5 mL Eppendorf microcentrifuge tubes or paper envelopes (7 cm × 10 cm), previously autoclaved under the same conditions. Multiple batches were prepared for each fungal strain, with six to ten tubes per isolate. The labeled tubes or envelopes, indicating the strain number and date, were then sealed inside low-density polyethylene bags (9 cm × 12 cm; Carlos Gonzalez S.A., Haedo, Buenos Aires, Argentina). All samples were stored at 4 ± 2 °C in a refrigerator as stock cultures until further use (Table 1).

Recovery of cultures

To recover the cultures, two to three strips of filter paper were placed (using sterilized forceps) in an adequate sterilized culture medium, potato dextrose agar (PDA) or Sabouraud dextrose agar +1% yeast extract (SDAY 1%) according to the species, contained in sterilized Petri dishes (60 mm diameter). Cultures were incubated at 25 ± 1 °C in darkness until fungal sporulation (Fig. 1). Cultures were monitored for viability and/or contamination.

Morphological stability assessment and viability

For each evaluation of cultures, isolates were randomly retrieved from the collection, both in terms of species and preservation time, to ensure representative testing. In total, four isolates each of *Beauveria*, *Metarhizium*, and *Cordyceps*, three of *Akanthomyces*, one of *Lecanicillium*, and two of *Purpureocillium* were analyzed. To assess strain stability following preservation, culture characteristics were considered, such as the analysis of culture morphology on plate, the abundance of sporulation, the presence or absence of sectors or abnormal growth, the presence of contamination by other fungi and/or bacteria, and whether they maintained their original morphological characteristics; these characteristics were compared with those exhibited by the original strain.

Viability of the cultures was calculated according to the modified method proposed by Lane et al.[9]. Conidial viability was determined by measuring the percentage of germination; 100 µL of adequate culture media was added to sterile glass microscope slides that were placed inside Petri dishes with filter paper moistened with a few drops of distilled water (viability chamber). This provided humidity for fungal conidia germination. Culture medium was inoculated using a semi-automatic micropipette at a concentration of 1×10^5 conidia/mL and kept in darkness at 25 ± 1 °C for 24 h. Germination was quantified for at least 200 conidia from each chamber. Conidia were considered to have germinated when they produced a germ tube that was at least half the length of the conidia. Three replicates per isolate were performed, and the mean number of germinated conidia out of the total considered was calculated.

Virulence bioassays

Virulence was tested using the insect species that were available after the conidia storage periods (Table 1). Specifically, isolates of *Beauveria* and *Metarhizium* were evaluated by the immersion method on healthy larvae of *Tenebrio molitor* (Coleoptera: Tenebrionidae) reared in artificial colonies[7]. Other genera (Table 1) were not included in these assays because some isolates did not resume growth after storage. Moreover, *T. molitor* is not susceptible to these fungi. Groups of five *T. molitor* were exposed to a 1×10^8 conidia/mL suspension in an 80 mL beaker for 30 s. The insects were then transferred to plastic cups (250 cc) at 25 ± 1 °C and 70% ± 5% RH. Wheat bran, used as the larval diet, was placed inside the containers and changed every 2 d. A total of ten larvae of *T. molitor* per replicate was used. Two replicates of each treatment were conducted. The experiment was repeated twice at different times. Mortality was monitored daily until 20 d after treatment. Dead insects were removed daily and placed on slides inside Petri dishes (100 mm diameter) containing filter paper moistened with sterile distilled water. Petri dishes were sealed with PARAFILM® and kept in an incubator chamber at 25 ± 1 °C and 70% ± 5% RH. Mycelia emergence was monitored for a total of 7 d. Infected insects with evidence of external fungal growth were examined under a stereomicroscope,

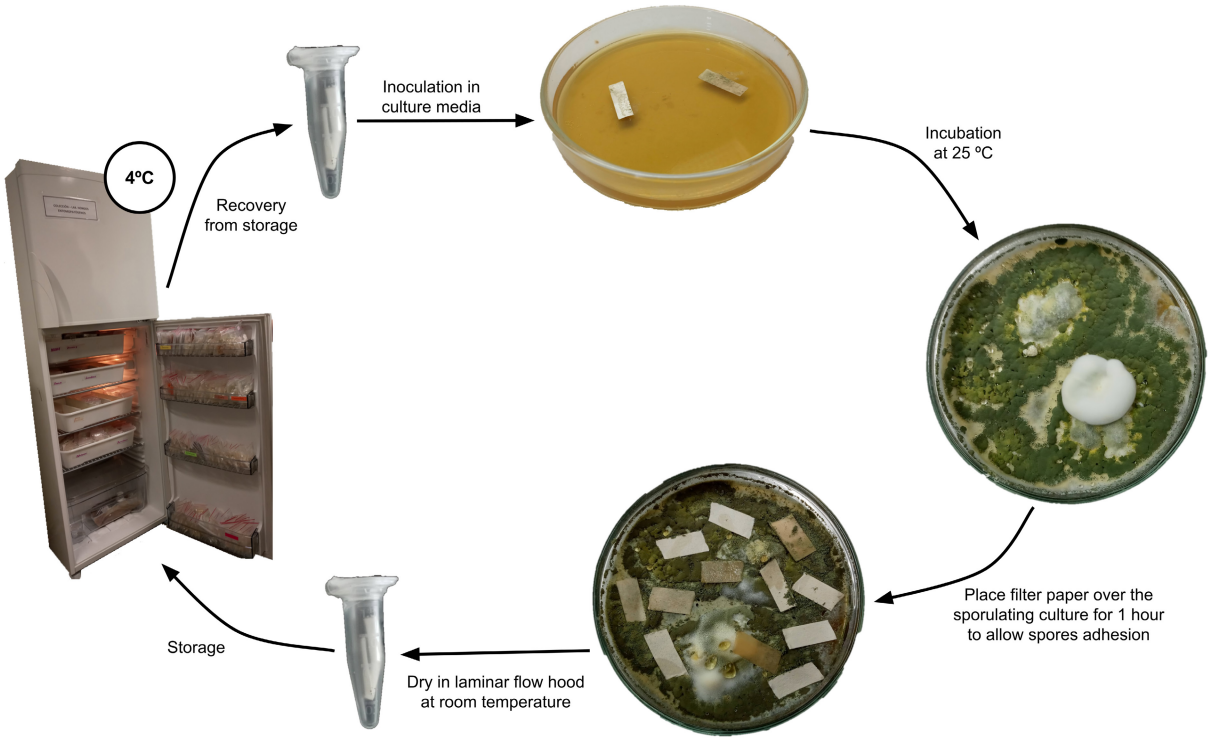

Fig. 1 Schematic representation of the paper-based preservation method for EPF.

and the fungus was re-isolated in culture medium to confirm the cause of infection.

Results and discussion

Although the successive subculturing method is commonly used for maintaining fungal cultures for commercialized strains, as noted by Brownbridge et al.[10], in the laboratory, this approach was ineffective for strains, likely representing an exception rather than the rule. The filter paper method was found to be effective for preserving and storing isolates of *Akanthomyces*, *Beauveria*, *Lecanicillium,* and *Metarhizium* (Table 1). *Beauveria* and *Metarhizium* species assessed in this study showed variable viability periods ranging from two to nine years, depending on the isolate. This highlights the variability in culture stability among entomopathogenic fungi isolates[10]. Viability of *B. bassiana*, *M. anisopliae,* and *M. hybridum*[11,12] conidia on filter paper was above 70% for all strains. Table 1 shows that all *B. bassiana*, *M. anisopliae,* and *M. hybridum* strains were able to maintain a high degree of pathogenicity after recovery. Two *Akanthomyces* and one *Lecanicillium* strains showed good growth and abundant sporulation in the culture medium after recovery. Furthermore, they maintained viability above 95% germination for three to seven years after storage, depending on the strain (Table 1). Only one isolate of *A. lecanii* showed fungal contamination (Table 1). *Purpureocillium* sp. strains showed good growth and sporulation in the culture medium, but *in vitro* conidia germination varied among the different strains (Table 1). Although both *Purpureocillium* (CEP780) and *Cordyceps fumosorosea* (CEP371) exhibited good hyphal growth and sporulation, their conidia germination was unexpectedly low, with germination rates below 35% (Table 1). Cases such as these demonstrate that conidia may grow and sporulate while losing viability, which shows that considering only growth and sporulation can provide an incomplete assessment. In fact, some preservation studies have focused primarily on growth and sporulation[5]. These results highlight that viability should also be considered an important criterion to take into account, as it provides relevant information. In this study, the preservation period of each isolate is provided in Table 1; however, not all intermediate times are represented because isolates were chosen randomly. This strategy allowed the testing of the method across a representative diversity of species and storage durations. When evaluating a preservation method, it is advisable to establish a testing program to ensure strain stability, in which known properties are periodically verified[13].

The fungal cultures used for this preservation method should correspond to the first subculture following primary isolation, as the initial isolation medium contains antibiotics. Preserving cultures exposed to antibiotics should be avoided to prevent potential genetic alterations. Additionally, no more than two or three subsequent transfers should be performed to maintain sporulation capacity, infectivity, and viability, as previously mentioned.[2,14]. Moreover, since growth and morphology do not distinguish between viable and non-viable conidia, it is necessary to further determine conidia viability *in vitro* to validate the preservation methods selected for EPF. The filter paper method was not very effective in the preservation of *Cordyceps* sp. strains. For example, CEP371 showed good growth and sporulation, but loss of viability, while CEP307 showed good mycelial growth but loss of sporulation, or the strains did not grow and died (Table 1). These observations are consistent with findings from Ayala-Zermeño et al.[5], who reported that *Cordyceps* spp. could be effectively preserved for up to two years using cryopreservation, lyophilization, or nitrogen storage, but not under water or mineral oil, where viability is significantly reduced. This supports the idea that preservation methods involving reduced metabolic activity are better suited for long-term storage, while more metabolically active approaches, such as paper-based preservation, may be more appropriate for short-term applications and species with greater tolerance. The method used here was adapted from the work of Fong et al.[8], who used vacuum

drying and storage at −20 °C for preserving various fungi (not entomopathogenic species). In this study, drying was performed in a laminar flow hood, and storage was carried out at 4 °C. These modifications were made to avoid the risk of residual moisture freezing at −20 °C, which could damage the fungal cultures through ice crystal formation. However, this adaptation likely resulted in less uniform drying and may explain the greater variability in viability observed among the tested strains, particularly in more sensitive genera such as *Cordyceps*. In all the strains assessed, bacterial or fungal contamination was very low; furthermore, all the recovered cultures were found to be free of mite infestation. Morphological stability, including cultural traits, sporulation, viability, and pathogenicity, is important as an indirect indicator of genetic stability. It is essential for decision-making in EPF culture collections, as these parameters provide rapid and practical indicators of strain performance. Although genetic analyses can offer valuable insights, especially when phenotypic changes are detected[5], molecular tools are often limited by higher costs and are not always required for initial screening. It is also important to note that some phenotypic alterations, such as loss of sporulation or reduced growth, may result from physiological or nutritional deficiencies rather than genetic instability. Therefore, genetic analyses must be carefully designed to generate information that is directly applicable to decision-making. There is still much to explore in the development and application of molecular tools that can support practical decisions in EPF preservation and quality control.

The advantages of the evaluated method are the practical, easy, and economical way to be developed, for use in routine bioassays and short-term stock maintenance, as well as the simple way to be delivered or transported to other laboratories in other locations. The cost of implementation is very low, and no sophisticated or specialized equipment is required when compared to lyophilization or ultra-low temperature methods (e.g., −196 °C using liquid nitrogen or −80 °C freezers). While lyophilization and cryopreservation in liquid nitrogen are excellent and long-lasting techniques, they are also costly to implement, involve a very high initial investment, and are labor-intensive[4]. Consequently, these methods are not accessible to all laboratories due to their high cost.

The methods using filter paper for the preservation of cultures mostly referred to bacteria[15]. However, Fong et al.[8] described the filter paper method for the successful storage of fungal cultures, referring to a maximum of four years of preservation at −20 °C. This protocol differs from the one described by Fong et al.[8] in the preservation temperature and in the drying of the cultures. The results obtained show that the filter paper method is effective in preserving conidial EPF for up to a maximum of nine years of preservation, depending on the species and strain. Comparing the described method with other simple ones, such as water, glycerol −20 °C, mineral oil, and silica gel[3,5], these reach similar results in terms of preservation time for EPF. When evaluating the preservation of EPF, it is essential to assess not only the macroscopic characteristics of the culture, such as growth and sporulation, but also viability, as an indirect measure of virulence, since germ tube formation facilitates cuticle penetration. As described by Faria et al.[16], accurate estimation of conidial viability is crucial. Their findings highlight that reliable viability assessment enhances the predictive value of virulence, reinforcing its relevance when selecting appropriate preservation techniques. In many cases, it has been observed that after preservation, cultures exhibit growth and sporulation but have very low viability, requiring the exclusion of that preservation method.

In conclusion, to determine the suitability of a preservation method for EPF, both macroscopic characteristics and viability must be evaluated. The present study suggests that the preservation of EPF using filter paper is a suitable method for fungi such as *Akanthomyces* sp., *Beauveria* sp., *Lecanicillium* sp., *Metarhizium* sp., and *Purpureocillium* sp. However, for *Cordyceps* sp., the preservation method shows low efficiency and is considered only as a short-term alternative (no more than one or two years, depending on the strains). Storage of EPF without great loss of viability and pathogenicity is a major concern for their preservation in laboratories, culture collections, and the industrial sector for bioinput production. In addition to culture viability, pathogenicity tests for *Beauveria* and *Metarhizium* demonstrate that the filter paper method was successful for periods ranging from two to nine years, respectively (Table 1). This method is effective for the preservation of conidial EPF in many cases, but its efficiency may depend on the species. Laboratories with modest resources can achieve scientific services with high standards, and sophisticated equipment is not a prerequisite for good microbiological practices. There are no universal methods for the preservation of microbial cultures; it is always necessary to balance the advantages and disadvantages of different methods, as it is impossible to avoid causing undesirable collateral damage to cultures[17–19]. This is the first report on the use of filter paper as a method of preserving EPF at 4 ± 2 °C.

Ethical statements

Not applicable.

Author contributions

The authors confirm contributions to the paper as follows: study conception and design: Gutierrez AC; data collection: Gutierrez AC, Scelsio NS; analysis and interpretation of results: Gutierrez AC, Scelsio NS, Lozano F, Hipperdinger ML; draft manuscript preparation: Gutierrez AC, Scelsio NS, Lozano F. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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